Tranilast attenuates connective tissue growth factor-induced extracellular matrix accumulation in renal cells

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Tranilast (*N*-[3,4-dimethoxycinnamoyl]anthranilic acid) is a synthetic compound that we have recently reported to inhibit transforming growth factor-β1 (TGF-β1)-induced tubulointerstitial fibrosis in the kidney. Connective tissue growth factor (CTGF) is recognized as a potent downstream mediator of TGF-β1. Both proximal tubule cells (PTCs) and cortical fibroblasts (CFs) are considered to be responsible for the production of tubulointerstitial extracellular matrix (ECM). These studies were undertaken to assess the profibrotic effects of CTGF in an *in vitro* model of the human PTCs and CFs, and to determine whether tranilast is effective in limiting the *in vitro* matrix responses induced by CTGF. Primary cultures of PTCs and CFs were exposed to CTGF (20 ng/ml) $\pm$ tranilast (100 μM). Cell hypertrophy and the secretion of the ECM proteins fibronectin and collagen IV were determined. The effects of tranilast on TGF-β1-induced CTGF mRNA expression and on phosphorylation of Smad2 were determined. CTGF significantly induced cell hypertrophy, increased fibronectin, and collagen IV secretion in PTCs and CFs. In all cases, the CTGF-induced increase in ECM protein was inhibited in the presence of tranilast. Tranilast reduced CTGF mRNA and phosphorylation of Smad2, which were induced by TGF-β1 in PTCs and CFs. These results suggest that tranilast is a potential effective antifibrotic compound in the kidney, exerting its effects via inhibition of TGF-β1-induced CTGF expression and downstream activation of the Smad2 pathway in both PTCs and CFs.

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The tubulointerstitium of the kidney accounts for approximately 90% of the kidney volume. Injury to the tubulointerstitium, resulting in expanded extracellular matrix (ECM) and tubular atrophy, is the key structural determinant of progressive renal disease independent of the primary pathology.[1,2] Proximal tubule cells (PTCs) and cortical fibroblasts (CFs) are the cellular elements primarily involved in the pathological changes in human renal fibrosis.[2,3]

Transforming growth factor-β1 (TGF-β1) and more recently connective tissue growth factor (CTGF) have been recognized as important cytokines involved in renal fibrosis. CTGF is considered to be a key downstream mediator of the profibrotic effects of TGF-β1. It is clear that both PTCs and CFs produce significant amount of TGF-β1 under basal conditions,[4,5] which is amplified in disease states and correlates with clinical and histological markers of progressive pathology.[6] We have recently reported that CTGF requires TGF-β1, signalling through the TβRII in PTCs and CFs, to exert its fibrogenic response in this *in vitro* model.[7]

Tranilast (*N*-[3,4-dimethoxycinnamoyl]anthranilic acid) is a drug initially used for its 'antiallergy' effects, where it is thought to act as a mast cell-stabilizing agent.[8] It has also been used in disorders such as scleroderma, which are associated with an excessive fibrotic response.[9] In a small pilot study in patients with advanced diabetic nephropathy, tranilast was reported to slow the decline in glomerular filtration rate over a 12-month period.[10] More recently, we have demonstrated in a rat model of experimental diabetes mellitus that concurrent treatment with tranilast attenuates both albuminuria and tubulointerstitial pathology. In this study, we further demonstrated that tranilast blocked the enhanced synthesis of collagen and non-collagen proteins induced by TGF-β1 in CFs.[11] CTGF is increasingly reported as having a pathogenic role in diabetic complications, including nephropathy. However, whether the pro-fibrotic effects of CTGF can be modulated by tranilast in either PTCs or CFs is unknown. Furthermore, the effects of tranilast on TGF-β1-induced CTGF mRNA and TGF-β-induced Smad signaling in both PTCs and CFs have not been studied.

RESULTS

Cell cytotoxicity

Exposure of PTCs to 100 μM had no significant effect on the levels of formazan release compared to control levels ($106 \pm 2.0\%$; $n = 4$). Similarly, exposure to CFs to 100 μM had no significant effect on the levels of formazan release compared to controls ($93 \pm 0.9\%$, $n = 4$). These data indicate that any effects of tranilast treatment in the current study cannot be attributed to cytotoxicity.

Tranilast inhibits CTGF-induced cellular hypertrophy in PTCs and CFs

Exposure of PTCs to CTGF caused a significant increase in cell protein content per cell to $133 \pm 6.7\%$ ($P < 0.0001$) compared to control values (Figure 1a). Similarly in CFs, exposure to CTGF induced a significant increase in cell protein content per cell to $141 \pm 8.7\%$ ($P < 0.0001$) of control (Figure 1b). The hypertrophic effect of CTGF in both PTCs and CFs was inhibited when the cells were incubated with CTGF in the presence of tranilast.

Tranilast attenuates CTGF-induced matrix synthesis in PTCs and CFs

The increase in cellular hypertrophy induced by CTGF in PTCs and CFs was paralleled by an increase in the levels of the markers of matrix deposition. Exposure of PTCs to CTGF, respectively, increased fibronectin and collagen IV secretion to 155 ± 22 and $477 \pm 124\%$ (both $P < 0.005$) of control levels (Figure 2a and b). Similarly in CFs, exposure to CTGF, respectively, increased fibronectin and collagen IV secretion to $148 \pm 8.9\%$ ($P < 0.005$) and $309 \pm 67\%$ ($P < 0.05$) of control levels (Figure 2c and d). The increased production of fibronectin and collagen IV secretion by PTCs and CFs in the presence of CTGF were inhibited in the presence of tranilast.

Tranilast reduces TGF-β1-induced CTGF mRNA expression

We have previously demonstrated that TGF-β1 stimulated CTGF with a peak induction at 24 h.[7] Hence, experiments were undertaken after exposure to the defined conditions for 24 h. PTCs and CFs exposed to 2 ng/ml TGF-β1 for 24 h induced a respective 2.5 ± 0.3 ($P < 0.005$) and 2.5 ± 0.2 ($P < 0.005$) fold increase in CTGF mRNA expression compared to control value. In both cell types, this induction was inhibited by tranilast (Figure 3a and b).

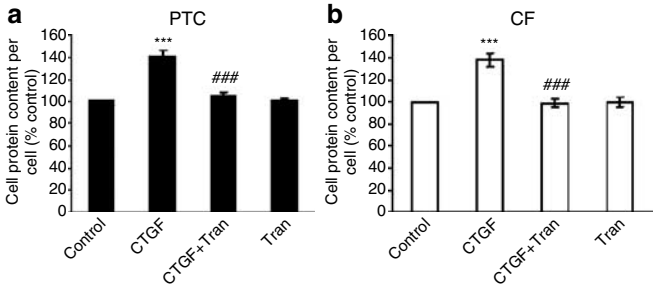

Figure 1 | Tranilast inhibits CTGF-induced cellular hypertrophy. (**a**, black bars) PTCs and (**b**, open bars) CFs were exposed to control, 20 ng/ml CTGF, 20 ng/ml CTGF plus 100 μM tranilast, or 100 μM tranilast alone for 48 h. Results are expressed as mean $\pm$ s.e.m. and are standardized as a percentage of the control values. $^{***}P < 0.0001$ vs control; $^{\#\#\#}P < 0.0001$ vs CTGF; $n = 6$.

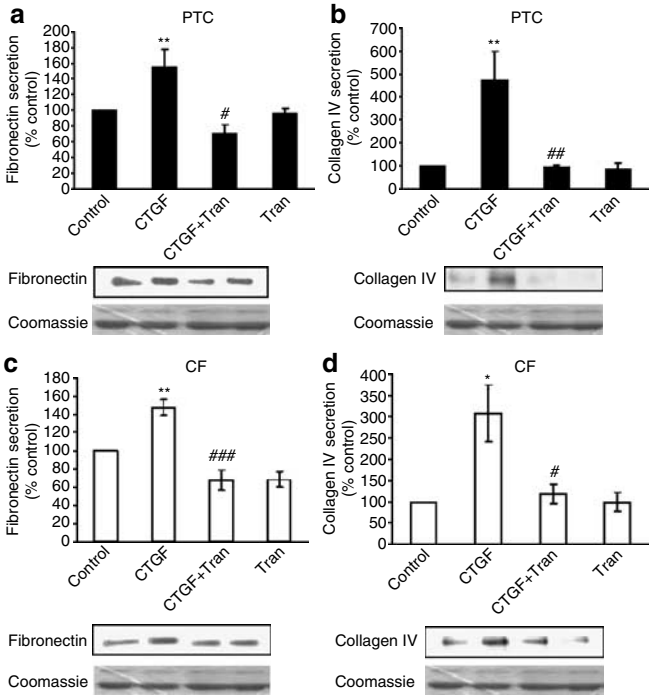

Figure 2 | Tranilast attenuates CTGF-induced matrix synthesis. (**a**, black bars) PTCs and (**b**, open bars) CFs were exposed to control, 20 ng/ml CTGF, 20 ng/ml CTGF plus 100 μM tranilast, or 100 μM tranilast alone for 48 h. (**a** and **c**) Fibronectin and (**b** and **d**) collagen IV secretion were measured. Representative Western blots for fibronectin, collagen, and Coomassie Brilliant Blue staining, which served as a loading control, are shown. Results are mean $\pm$ s.e.m. and are standardized as a percentage of the control values. $^{**}P < 0.005$; $^{*}P < 0.05$ vs control; $^{\#\#\#}P < 0.0001$; $^{\#\#}P < 0.005$, and $^{\#}P < 0.05$ vs CTGF; $n = 6$.

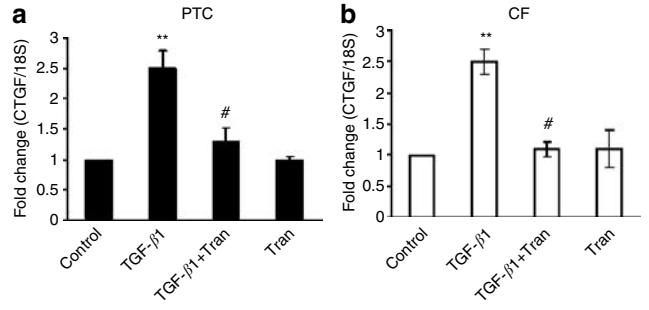

Figure 3 | Tranilast inhibits TGF-β1-induced CTGF mRNA expression. (**a**, black bars) PTCs and (**b**, open bars) CFs were exposed to control, 2 ng/ml TGF-β1, 2 ng/ml TGF-β1 plus 100 μM tranilast, or 100 μM tranilast alone for 24 h. CTGF mRNA expression was measured by real-time PCR. Real-time PCR results were normalized to housekeeping gene 18S and shown as fold change compared to control value. $^{**}P < 0.005$ vs control and $^{\#}P < 0.05$ vs TGF-β1; $n = 6$.

Tranilast inhibits TGF-β-induced cellular hypertrophy and matrix synthesis

We have recently demonstrated that tranilast blocked the enhanced synthesis of collagen and non-collagen proteins induced by TGF-β1 in CFs.[11] To further confirm this inhibitory effect on PTCs as well, PTC exposed to 2 ng/ml TGF-β1 resulted in a significant increase in cell protein content per cell to 171±8.7% (Figure 4a) and 157±10.0% (Figure 4b) (both $P<0.0001$) compared to control values. The hypertrophic effect of TGF-β1 was inhibited when the cells were incubated with TGF-β1 in the presence of tranilast. Tranilast alone had no effect on cellular protein content in PTCs and CFs.

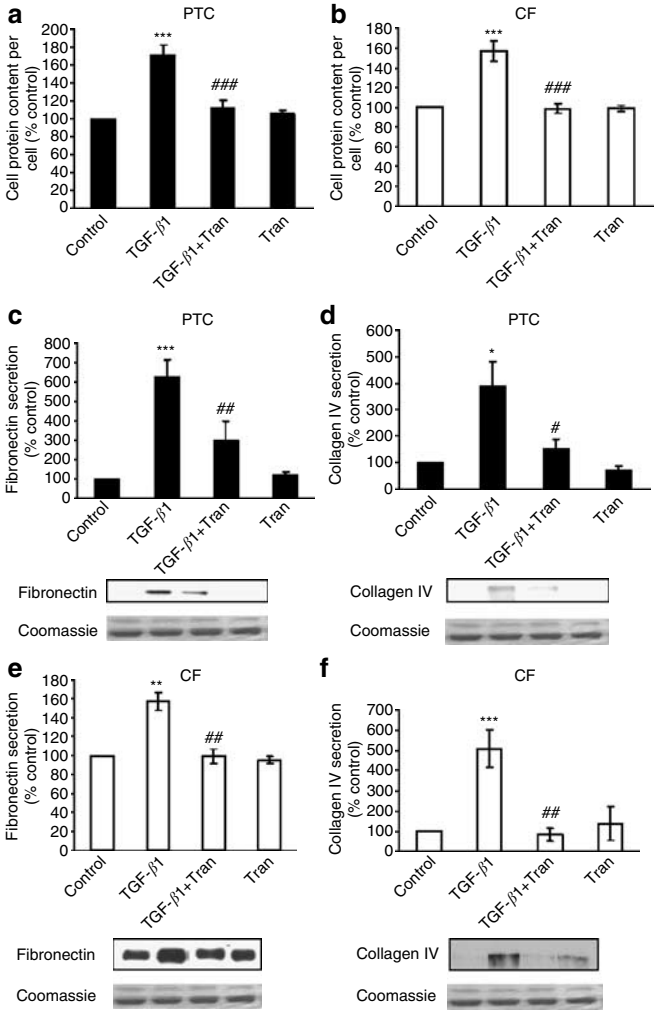

Figure 4 | Tranilast inhibits TGF-β1-induced cellular hypertrophy and matrix synthesis in both PTCs and CFs. (Black bars) PTCs and (open bars) CFs were exposed to control, 2 ng/ml TGF-β1, 2 ng/ml TGF-β1 plus 100 μM tranilast, or 100 μM Tranilast alone for 48 h. (**a** and **b**) Cell protein content normalized to cell number was used as a marker of cell hypertrophy. (**c** and **e**) Fibronectin served as a marker of non-collagenous protein and (**d** and **f**) collagen IV served as marker of collagen synthesis. Representative Western blots for fibronectin, collagen IV, and Coomassie Brilliant Blue staining, which served as a loading control, are shown. Results are mean±s.e.m. and are standardized as a percentage of the control values. $^{***}P<0.0005$ vs control; $^{\#\#\#}P<0.0005$ vs TGF-β1; $n=6$.

The cellular hypertrophy induced by TGF-β1 in PTCs and CFs was paralleled by an increase in the levels of the markers of matrix deposition. In PTCs, exposure to TGF-β1 increased fibronectin and collagen IV secretion to 625±92% ($P<0.0001$) and 390±90% ($P<0.05$), respectively, of control levels (Figure 4c and d). The increases in fibronectin and collagen secretion were inhibited in PTCs co-exposed to TGF-β1 and tranilast. Exposure to tranilast alone had no effect on these parameters. Similarly in CFs, exposure to TGF-β1 increased fibronectin and collagen IV secretion to 157±9.6% ($P<0.005$) and 510±94% ($P<0.0001$), respectively, of control levels. The increases in fibronectin and collagen secretion were inhibited in CFs exposed to both TGF-β1 and tranilast.

Tranilast inhibits TGF-β-induced phosphorylation of Smad2

We have previously demonstrated that TGF-β1 induced pSmad2 with a peak induction at 30 min.[7] Hence, these experiments were performed after 30 min exposure to the defined conditions. TGF-β1 induced pSmad2 in PTCs and CFs within 30 min, which was inhibited by tranilast (Figure 5a and c). These data suggest that tranilast inhibits the profibrotic effect of TGF-β1 via a reduction in pSmad signaling.

We have also demonstrated that CTGF did not detectably induce pSmad2 until 24 h.[7] Hence, experiments were performed at 24 h exposure to the defined conditions. These demonstrated that tranilast inhibits CTGF-induced pSmad2 expression (Figure 5b and d), which may be explained by upstream abrogation of the action of TGF-β1.

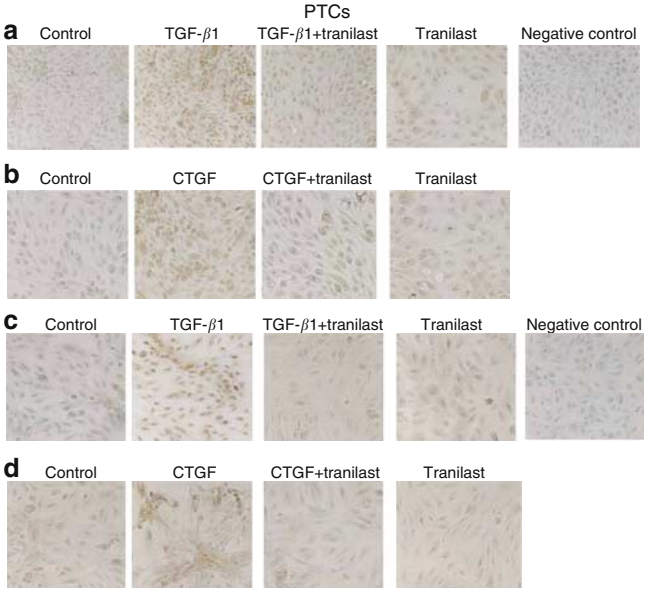

Figure 5 | Tranilast inhibits TGF-β-induced phosphorylation of Smad-2. (**a** and **c**) PTCs and CFs were exposed to control, 2 ng/ml TGF-β1, 2 ng/ml TGF-β1 plus 100 μM tranilast, or 100 μM Tranilast alone for 30 min. Similarly in CTGF, (**b**) PTCs and (**d**) CFs were exposed to control, 20 ng/ml CTGF, 20 ng/ml CTGF plus 100 μM tranilast, or 100 μM tranilast alone for 24 h.

Table 1 | Quantitation of pSmad2 in (A) PTCs and in (B) CFs

Treatment	Positive staining cells (%)	Score	*P*-values
(A) Quantitation of pSmad2 in PTCs			
Control-A	8.7 ± 0.3	0	
TGF-> β1	92.7 ± 1.5	4+	> P < 0.0001 (vs control-A)
TGF-> β1+tranilast	10.0 ± 2.6	1+	> P < 0.0001 (vs TGF-> β1)
Tranilast-A	8.0 ± 0.6	0	> P > 0.05 (vs control-A)
Control-B	8.0 ± 0.6	0	
CTGF	85.3 ± 0.3	4+	> P < 0.0001 (vs control-B)
CTGF+tranilast	7.0 ± 1.5	0	> P < 0.0001 (vs CTGF)
Tranilast-B	6.7 ± 1.2	0	> P > 0.05 (vs control-B)
(B) Quantitation of pSmad2 in CFs			
Control-C	4.0 ± 0.6	0	
TGF-> β1	94.7 ± 1.5	4+	> P < 0.0001 (vs control-C)
TGF-> β1+tranilast	9.7 ± 1.2	0	> P < 0.0001 (vs TGF-> β1)
Tranilast-C	3.3 ± 0.9	0	> P > 0.05 (vs control-C)
Control-D	5.3 ± 0.9	0	
CTGF	78.0 ± 1.5	4+	> P < 0.0001 (vs control-D)
CTGF+tranilast	4.7 ± 0.9	0	> P < 0.0001 (vs CTGF)
Tranilast-D	6.0 ± 1.2	0	> P > 0.05 (vs control-D)

CF=cortical fibroblast; CTGF=connective tissue growth factor; PTC=proximal tubule cell; TGF-β1=transforming growth factor-β1; tranilast=*N*-[3,4-dimeth-xycinnamoyl]anthranilic acid.
N.B.: Score calculation: <10% positive cells=0, 10–25%=1+, 25–50%=2+, 50–75%=3+, and 75–100%=4+.

Data were assessed using a quantitative scoring method as described in the Materials and Methods section and shown for PTCs in Table 1A and for CFs in Table 1B.

DISCUSSION

The present study provides further information to suggest that tranilast is an effective antifibrotic strategy in progressive renal disease. We have demonstrated that tranilast prevents CTGF-induced matrix production in human renal PTCs and CFs, which is similar to the antifibrotic effect previously observed in CFs treated with TGF-β1.[11] In the present study, we have additionally confirmed that tranilast effectively blocks TGF-β1-induced matrix production in PTCs. Based on our previous and current study findings, we hypothesize that tranilast limits the antifibrotic effects of CTGF by inhibiting TGF-β1 and downstream activation of pSmad2. These *in vitro* findings suggest the basis for our recent demonstration that tranilast ameliorates objective markers of fibrosis in an animal model of diabetic nephropathy.[11] Clearly, TGF-β1 and CTGF have been implicated in the development of diabetic nephropathy.[12–14] However, the effect of tranilast on the manifestation of diabetic nephropathy, that is, excessive tubulointerstitium matrix production has not previously been demonstrated. Tranilast has previously been shown to block TGF-β1-induced fibrosis in the skin.[15,16] The current study supports its use as an antifibrotic agent in circumstances where profibrotic cytokines other than TGF-β1 are implicated.

The pharmacological effects of tranilast have not to date been widely appreciated. Tranilast is an immunomodulatory drug that inhibits the release of cytokines and chemical mediators from various cells, including macrophages and fibroblasts. Our data are supported by others who demonstrated that tranilast suppresses hypertrophic scarring by inhibiting collagen synthesis in fibroblasts, vascular smooth muscle cells, and macrophages.[10,15,17]

Initial reports in small numbers of patients suggested that tranilast slows the progression of advanced diabetic nephropathy and treatment with tranilast may suppress the accumulation of collagen in renal tissue. Hence, the therapeutic value in advanced diabetic nephropathy has been suggested.[10] It has also been reported that tranilast suppresses the elevation in mRNAs encoding the TGF-β type I and type II receptor in balloon-injured arteries in a dose-dependent manner.[18] The mechanism has not been elucidated, but it has been speculated that tranilast may act through the inhibition of receptor tyrosine kinase and possibly receptor serine threonine kinase activities.[19]

The blockade of the hypertrophic and pro-fibrotic effects of CTGF by tranilast is consistent with the suggestion that CTGF-induced renal fibrosis is mediated by facilitating the action of TGF-β1. Several studies have shown that tranilast inhibits TGF-β1 expression[18,20,21] and TβR II-induced phosphorylation of the type 1 receptor.[18] Our findings that tranilast inhibits TGF-β1-induced phosphorylation of Smad2 is consistent with these studies. Indeed, we have previously demonstrated that tranilast inhibits TGF-β-induced matrix production *in vitro* in human CFs.[11] We cannot exclude the possibility that tranilast may block integrins or other as yet unidentified intracellular receptors that might serve as CTGF receptors.[22–25] However, the hypothesis that CTGF promotes fibrosis through TGF-β1-dependent mechanisms is supported by a report from Abreu *et al.*[22] and our recent work,[7] which showed that CTGF may function as a chaperone to modify the conformation or solubility of TGF-β1, and thus facilitate TGF-β1 binding to the TβRII. The present study is consistent with this view, as we have demonstrated that tranilast inhibits TGF-β1-induced CTGF mRNA expression

and TGF-β1-induced pSmad2. Hence, the molecular mechanisms of tranilast as an antifibrotic agent on TGF-β1 and CTGF have been further explored.

In smmary, tranilast limits the observed increase in matrix protein produced by PTCs and CFs in response to the profibrotic cytokines TGF-β1 and CTGF, which are clearly implicated in progressive renal disease. It has no observable toxic effects with respect to cell viability at its effective concentration *in vitro*. Many pharmacological agents proposed to limit collagen deposition in fibrotic diseases are clinically unacceptable because of toxicity, or efficacy. Tranilast appears to be free from these limitations and it is proven to have minimum long-term side effects.[16] These studies should form the basis for future studies as to its usefulness in the treatment of human disease associated with kidney fibrosis. Moreover, this is the first study, to our knowledge, to explore the molecular mechanisms of tranilast on TGF-β signalling and on TGF-β1-induced CTGF expression.

MATERIALS AND METHODS

Cell culture

Segments of macroscopically and histologically normal renal cortex were obtained under aseptic conditions from patients undergoing nephrectomy for small (<6 cm) tumors. Patients were accepted for inclusion into the study if there was no history of renal or systemic disease known to be associated with tubulointerstitial pathology, which was confirmed by subsequent histopathology. Written informed consent was obtained from each patient before surgery and ethics approval for the study was obtained from the Royal North Shore Hospital Human Research Ethics Committee.

The methods for primary culture of both PTC and CF are described in detail elsewhere.[4,26] In brief, the kidney cortex was dissected from the medulla, finely minced, and then digested by collagenase (383 U/mg from Worthington, NJ, USA) for 30 min at 37°C and passed through a 100 μm mesh. The filtrate was resuspended in 50 ml of 45% Percoll (Pharmacia, Uppsala, Sweden) and centrifuged at 20 000 r.p.m. at 40°C for 30 min. The lowermost tissue band containing highly purified PTCs and the top band containing CFs were carefully removed and washed. The PTC fragment pellet was resuspended in serum-free hormonally defined media consisting of 1:1 (vol:vol) Dulbecco's modified Eagle's media and Hams F-12 (Trace, Australia), supplemented with 10 ng/ml (1.64 nM) epidermal growth factor, 5 mg/ml human transferrin, 5 mg/ml (0.87 mM) bovine insulin, 0.05 mM hydrocortisone, 50 mM prostaglandin E1, 50 nM selenium, and 5 pM tri-iodothyronine (all from Sigma, USA). The CF pellet was resuspended in Dulbecco's modified Eagle's media and Hams F-12 containing 10% fetal calf serum (Invitrogen, Australia). Passage 2 cells were used for all experiments. The ultrastructure, growth, and immunohistochemistry of both PTC and CF have been well characterized in our laboratory and shown to reproducibly reflect the biology and physiology of their *in vivo* counterparts.[4,26]

Antibodies and recombinant proteins

TGF-β1 was purchased from Sigma (MO, USA) and recombinant human CTGF was a generous gift from FibroGen (CA, USA). Mouse monoclonal fibronectin antibody was from Neomarkers (CA, USA) and rabbit polyclonal collagen IV antibody was from Abcam (Cambridge, UK). Phospho-Smad2 antibody was purchased from Chemicon, USA.

Experimental protocol

Cells were grown to approximately 80% confluence and then quiesced by incubation for 24 h in basic media (Dulbecco's modified Eagle's media and Hams F-12 containing 5 ng/ml human transferrin). Subsequently, the cells were exposed to the various experimental conditions described below for a further 48 h, unless otherwise described. Both PTC and CFs were exposed to medium containing 5 mM glucose plus 0.01% bovine serum albumin under experimental conditions as defined below. The concentrations used in these experimental conditions were determined by initial dose–response curve in both PTCs and CFs using between 2 and 200 ng/ml of CTGF. This determined that 20 ng/ml achieved maximal expression of fibronectin with no cellular toxicity. The effective concentration of tranilast was determined by a dose–response curve using 30, 100, and 300 μM tranilast. The 100 μM dose was chosen as it had potent inhibitory effect on TGF-β1- and CTGF-induced matrix production, but no toxic effect was observed on the cells. This dose had been previously used in other models.[27,28]

Initial experiments performed in PTC assessed the effect of CTGF $\pm$ tranilast or tranilast alone in both PTCs and CFs on cellular protein content as a marker of cellular hypertrophy, and on fibronectin and collagen type IV secretion as markers of ECM production. Collagen type IV was chosen as marker of synthesis of collagen-based protein. Fibronectin was chosen as a non-collagenous protein that serves as a scaffold for the deposition of other proteins. Furthermore, it functions as a fibroblast chemoattractant and promotes their differentiation, which may be a crucial phenomenon in the pathogenesis of tubulointerstitial fibrosis.[24]

Subsequent experiments assessed the effects of tranilast in both PTC and CFs on TGF-β-induced CTGF mRNA expression, TGF-β, and CTGF-induced Smad signaling.

Measurement of cytotoxicity

Cell viability following exposure of 100 μM tranilast was assessed using the CellTiter 96 AQueous One Solution kit (Promega, USA). The absorbance of the formazan released in each sample read and the results standardized to control. The levels of formazan in the culture supernatant reflect mitochondrial activity, thus a reduction in formazan levels released by cells represents a cytotoxic effect.

Cellular hypertrophy

Cellular hypertrophy is reflected by an increase in protein per cell as described previously by our group and others.[29–31] Manual cell counts were performed on trypsinized cells using a hemocytometer. Cellular protein was quantitated using BioRad protein assay (Hercules, CA, USA). Total cellular protein content normalized for cell number was determined as a marker of cellular hypertrophy.

Fibronectin and collagen IV Western blotting

Fibronectin and collagen IV secretion were determined by Western blotting on cell culture supernatants. Samples were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis under reducing conditions. Proteins were then transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, UK). Nonspecific binding sites were blocked for 1 h (5% non-fat milk

and 0.1% Tween-20 in phosphate-buffered saline (PBS)), after which the membranes were incubated in primary fibronectin and collagen IV antibodies overnight at 4°C, followed by washing four times after which they were incubated with peroxidase-labelled secondary antibodies (Amersham Pharmacia Biotech, UK) for 1 h and again washed four times. The blots were then detected using ECL (Amersham Pharmacia Biotech, UK). The bands corresponding to fibronectin (220 kDa) and collagen IV (180 kDa) were quantitated using NIH Image software v1.60. Coomassie Brilliant Blue staining was used to confirm that an equal amount of protein was loaded in each lane.

Immunocytochemistry staining

PTCs and CFs were treated with TGF-β1 or CTGF $\pm$ tranilast or tranilast alone for 48 h as described in the Materials and Methods sections and fixed on coverslips using ice-cold 4% formalin for 30 min followed by three times washing in PBS. Cells were then permeabilized with 0.2% Triton X-100 for 20 min followed by three washing in PBS. Cells were treated with 4% hydrogen perioxide in PBS to quench the endogenous peroxidase activity and then the nonspecific background was blocked using serum-free protein block (Dako, USA). Cells were incubated with pSmad2 primary antibody (1:200) at room temperature for 1 h followed by three washing in PBS. LSAB2 system-HRP was used for detection of pSmad2 according the manufacturer's instructions (Dako, USA). Mayer's hematoxylin was used to counterstain the cells. A negative control (non-pSmad2 antibody) was performed. Coverslips were mounted on glass slides using Dako glycerol mounting media. Slides were blinded and three random fields were digitized using a Nikon microscope attached to a digital camera. Data was quantitated using quantitative scoring method as described by McDonald and Pilgram.[32] In total, 100 cells were randomly chosen and counted on each slide. Less than 10% positive staining cells was scored 0, 10–25% positive cells was scored 1+, 25–50% positive cells was scored 2+, 50–75% positive cells was score 3+, and 75–100% positive cells was scored 4+.

Real-time reverse transcription polymerase chain reaction

Real-time polymerase chain reaction (PCR) following reverse transcription was used to assess transcript levels of CTGF. To ensure equal loading of cDNA samples, concurrent PCR reactions for 18S was performed. Both water blank and non-reverse-transcribed RNA samples were used as negative controls. Briefly, total RNA was extracted using RNeasy Mini kit (Qiagen, Australia) according to the manufacturer's instructions. Total RNA (2 μg) was treated with DNase I (Invitrogen, USA) and then cDNA was synthesized using reverse transcriptase Superscript II RT (Invitrogen, USA). Specific primers for the use of SYBR Green were designed as follows: CTGF – forward, 5′-GGCTTACCGACTGGAAGAC-3′ and reverse, 5′-AGGAGGCGTTGTCATTGG-3′. 18S served as an internal control: forward, 5′-CGGCTACCACATCCAAGGAA-3′ and reverse, 5′-GCTGGAATTACCGCGGCT-3′. Primers specificity in real-time PCR reactions was confirmed using reverse transcription PCR. A 25 μl of real-time PCR reaction included Brilliant SYBR Green QRT-PCR Master Mix according to the manufacturer's instructions (Stratagene, USA). Real-time quantitations were performed on the Bio-Rad iCycler iQ system (CA, USA). The fluorescence threshold value was calculated using the iCycle iQ system software. The calculation of relative change in mRNA was performed using the delta–delta method,[33] with normalization for the housekeeping gene 18S.

Statistical analysis

All results are expressed as a percentage of the control values (100%), with the exception of real-time PCR results, which are expressed as a fold change compared to the control value. Experiments were in duplicates and performed in at least three different culture preparations, hence $n = 6$. Results are expressed as mean $\pm$ s.e.m. Statistical comparisons between groups were made by analysis of variance, with pairwise multiple comparisons made by Fisher's protected least-significant difference test. Analyses were performed using the software package, Statview version 4.5 (Abacus Concepts Inc., Berkley, CA, USA). *P*-values less than 0.05 were considered significant.

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